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Close Similarity Between Endogenous Ecotropic Virus of *Mus musculus molossinus* and AKR Virus

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By using seven different restriction endonucleases, the cleavage patterns of the unintegrated proviral DNA from an ecotropic murine leukemia virus isolated from *Mus musculus molossinus* were found to be identical to those of AKR virus. An AKR [^{3}H]DNA probe can be completely saturated with *M. musculus molossinus* and *M. musculus castaneus* DNAs, although the arrangement of viral sequences in *M. musculus molossinus* DNA differed from that of AKR virus. These studies indicate that an AKR-type ecotropic virus is present in some wild Asiatic mice.

Both ecotropic and xenotropic murine leukemia viruses (MuLV's) are known to be carried as endogenous chromosomal determinants in *Mus musculus* (8, 14, 21). Xenotropic sequences are ubiquitous among laboratory and wild mice, and they presumably represent viral sequences that became established in the germ line before speciation of *M. musculus* (22). In contrast, ecotropic virus is present in some laboratory mice and not others, and in some wild mice and not others (7). The mice that lack ecotropic virus by biological assays also lack a portion (approximately 20%) of the viral genome as detected by nucleic acid hybridization analyses (7, 16). It seems likely that the ecotropic viruses are not as ancient in evolution as xenotropic viruses. Indeed, it is possible that ecotropic sequences in laboratory mice are a consequence of exposure to viruses of other species during domestication or laboratory adaptation (1).

Viruses of the ecotropic class have previously been isolated from three types of wild-caught *M. musculus*: the Kyushu mouse *M. musculus molossinus*, of southern Japan (4, 15); *M. musculus castaneus*, of East Asia (S. K. Chattopadhyay and M. R. Lander, unpublished data); and certain wild mouse populations studied by Gardner et al. (9). These isolates have not been thoroughly characterized biologically or biochemically, nor has it been established whether they are carried as endogenous, chromosomal proviruses.

To gain more insight into the nature and relationship of ecotropic MuLV's of wild mice and inbred mice, we have done a number of studies to further characterize the endogenous ecotropic MuLV of *M. musculus molossinus*.

MATERIALS AND METHODS

Specimens of *M. musculus molossinus* were obtained from two sources. Frozen tissues were obtained from T. H. Yosida, of the National Institute of Genetics, Misima, Japan. These mice were caught in the wild on Yonakuri Island, Japan. Laboratory-reared *M. musculus molossinus* and *M. musculus castaneus* were obtained from Michael Potter, and virus-negative tissue culture cell lines were developed from the two strains. For *M. musculus molossinus*, DNAs were prepared both from the frozen tissue (designated *M. musculus molossinus*-2) from Japan and from the tissue culture cell line (designated as *M. musculus molossinus*-1). Pregnant AKR mice were purchased from Jackson Laboratory, and pregnant NIH (National Institutes of Health) Swiss mice were obtained from the NIH colony.

Two isolates of ecotropic virus of *M. musculus molossinus* origin were studied. The majority of work was done with a strain induced from the tissue culture cell line by 5-iododeoxyuridine treatment; this virus was carried through two cycles of limiting dilution purification. The other strain was supplied by Christine Kozak, who isolated it from the spleen of a laboratory-reared *M. musculus molossinus* from Jackson Laboratory.

Methods for isolation, propagation, and quantitation of ecotropic virus and determination of tropism have been described (10, 19, 23). Preparation of AKR virus, synthesis and characterization of ^{3}H-labeled complementary DNA, and methods for DNA and RNA extraction have been published (5, 7). Procedures for nucleic acid hybridization are described in the legend of Fig. 1.

RESULTS

Characterization of the *M. musculus molossinus* virus. *M. musculus molossinus* is a high-virus mouse in that MuLV's can be obtained readily from the tissues of young adult

TABLE 1. *Restriction endonuclease cleavage products of unintegrated linear Akv-1 and M. musculus molossinus ecotropic virus isolates*

Enzyme	Sizes of fragments (kbp) from[a]:	
	Akv-1 ecotropic	*M. musculus molossinus* ecotropic isolates[b]
*Eco*RI	8.8	8.8
*Hin*dIII	5.8, 3.0	5.8, 3.0
*Xho*I[c]	4.6, 4.3	4.6, 4.3
*Sal*I	4.7, 4.2	4.7, 4.2
*Sac*I	5.1, 3.8	5.1, 3.8
*Sma*I[c]	4.6, 1.7, 1.5, 0.5, 0.4, 0.1	4.6, 1.7, 1.5, 0.5, 0.4, 0.1
*Kpn*I[c]	3.9, 2.8, 1.4, 0.5, 0.1	3.9, 2.8, 1.4, 0.5, 0.1

[a] Unintegrated viral DNA was isolated by Hirt procedure from NIH 3T3 cells 16 to 18 h after infection (12, 18). After restriction endonuclease digestion, the completeness of which was monitored by the addition of wild-type λ DNA, the DNAs were electrophoresed in a 0.75% agarose gel at 40 V for 16 to 18 h, transferred to nitrocellulose filters (25), and hybridized with an AKR MuLV [^{32}P]cDNA probe that had been synthesized from virion RNA in a reaction catalyzed by avian myeloblastosis virus DNA polymerase and primed with calf thymus DNA oligomers. The 0.1-kilobase pair (kbp) fragments were not visualized directly. Their presence was inferred from the 0.6-kbp *Sma*I and *Kpn*I fragments seen in the 8.8-kbp circular DNA as described by Lowy et al. (17).

[b] Two isolates were used. One was derived from a cell line from the colony of M. Potter (*M. musculus molossinus*-1 in the text). A second isolate was kindly provided by C. Kozak and was isolated from *M. musculus molossinus* obtained from Jackson Laboratory.

[c] Restriction endonuclease maps of AKR virus for these three enzymes have recently been published (17).

mice and can be easily activated from tissue culture cells by using 5-iododeoxyuridine (our observation as well as personal communications with C. Kozak and J. Hartley).

In its biological behavior, the *M. musculus molossinus* ecotropic virus was like the endogenous ecotropic strains of laboratory mouse origin. That is, it is N tropic, ecotropic, and XC$^+$. On biochemical analysis it was also indistinguishable from AKR virus. First, RNA from purified *M. musculus molossinus* virus saturated 100% of the sequences of an AKR virus ^{3}H-labeled complementary DNA [cDNA] probe. Second, analysis of the size of fragments generated by restriction enzyme cleavage of unintegrated linear provirus DNA molecules of AKR virus and of both isolates of the *M. musculus molossinus* virus gave identical results with all seven enzymes tested (Table 1). Although the map orientations of the fragments generated from *M. musculus molossinus* virus DNA have not been compared with those of the AKR virus, there seems little doubt but that the *M. musculus molossinus* and AKR viruses have identical restriction maps for these seven enzymes.

Viral sequences in *M. musculus molossinus* DNA. Data on nucleic acid hybridization studies between DNAs of *M. musculus molossinus*, AKR/J, and wild *M. musculus* from three widely separated geographic regions and a single-stranded [^{3}H]cDNA synthesized from AKR virus are shown in Fig. 1 and 2 and, along with other data, are summarized in Table 2. As shown previously, DNAs from certain wild *M. musculus* do not fully saturate the AKR virus probe, but hybridize to about 80% of it (7). In contrast, 100% of the probe formed hybrids with DNA from *M. musculus molossinus* and *M. musculus castaneus*. Thus, with respect to the saturation hybridization results, *M. musculus molossinus* and *M. musculus castaneus* reacted like AKR, whereas the other wild *M. musculus* reacted like non-ecotropic virus-yielding laboratory mice such as NIH Swiss, C57L, NZB, and 129 (7).

Analysis of the thermal melting profiles of the hybrids formed between the ^{3}H-labeled AKR viral DNA probe and the cellular DNAs (see Fig. 1 insets and Table 1) showed that the probe formed well-matched hybrids with *M. musculus molossinus* and AKR DNAs (ΔTe_{50} [change in midpoint thermal elution value] = 2 to 2.6°C) but relatively poorly matched hybrids with the DNA from the wild-caught *M. musculus* ($\Delta Te_{50} \leq 6$ to 8°C). Thus, in respect to saturation hybridization and melting profile analyses, the cellular DNAs from AKR, *M. musculus molossinus*, and *M. musculus castaneus* yielded similar, if not identical, results.

However, when the data from association kinetics experiments (Fig. 1) were analyzed by a reciprocal plotting method (Fig. 2; 6, 26), distinctly different patterns emerged. Our previous studies on endogenous ecotropic genomes of laboratory mice showed that in those mouse strains where the complete AKR viral genome is present, there are two general classes of sequences with respect to their abundance in the cellular DNA (16). One set is present as 7 to 10 or more copies per haploid genome, whereas the other, less abundant set is present at one to two or three to four copies per haploid genome, depending on the strain.

Like AKR DNA, the *M. musculus molossinus* DNA showed a moderately abundant set of sequences and a set that gave a slope 3 to 4 (relative to that of unique cellular DNA reassociation). In both cases the line fitted to the second component intersected the ordinate at about 2.0, indicating that about half of the probe sequences were reacting with this population of

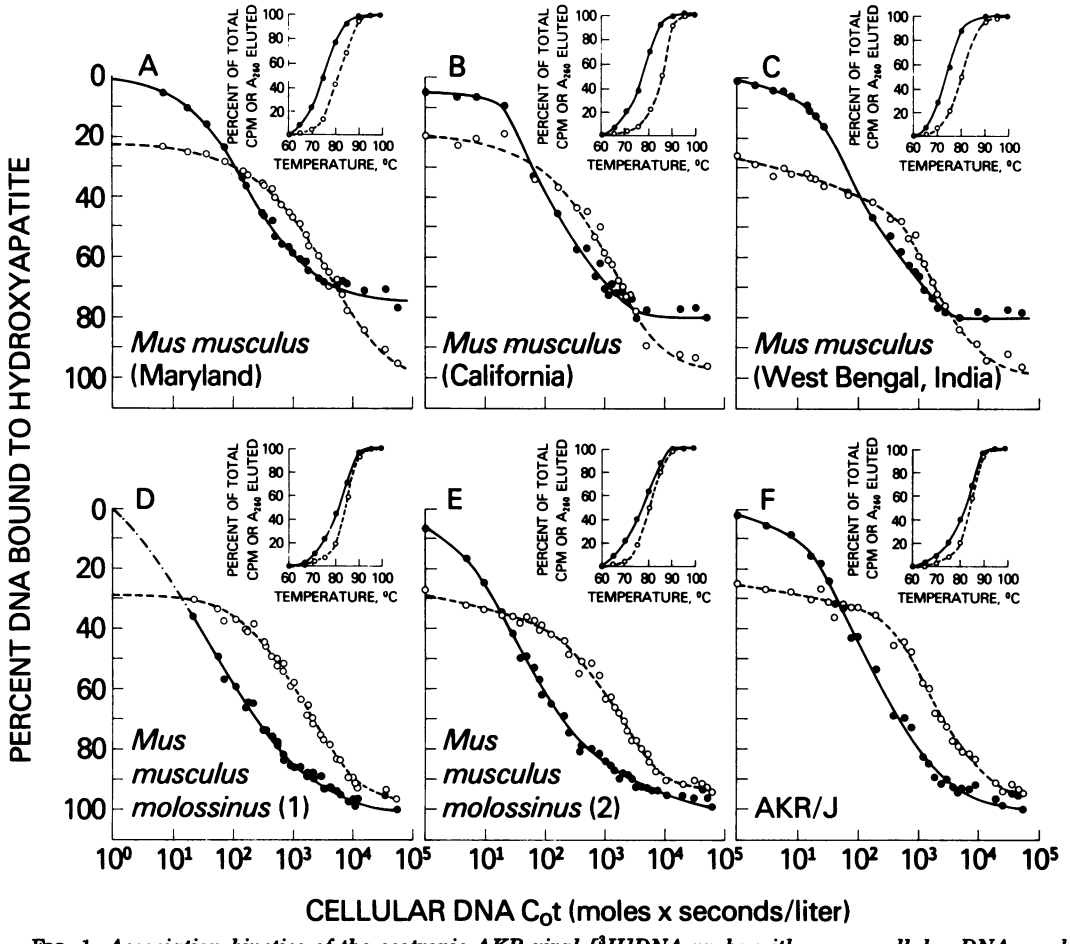
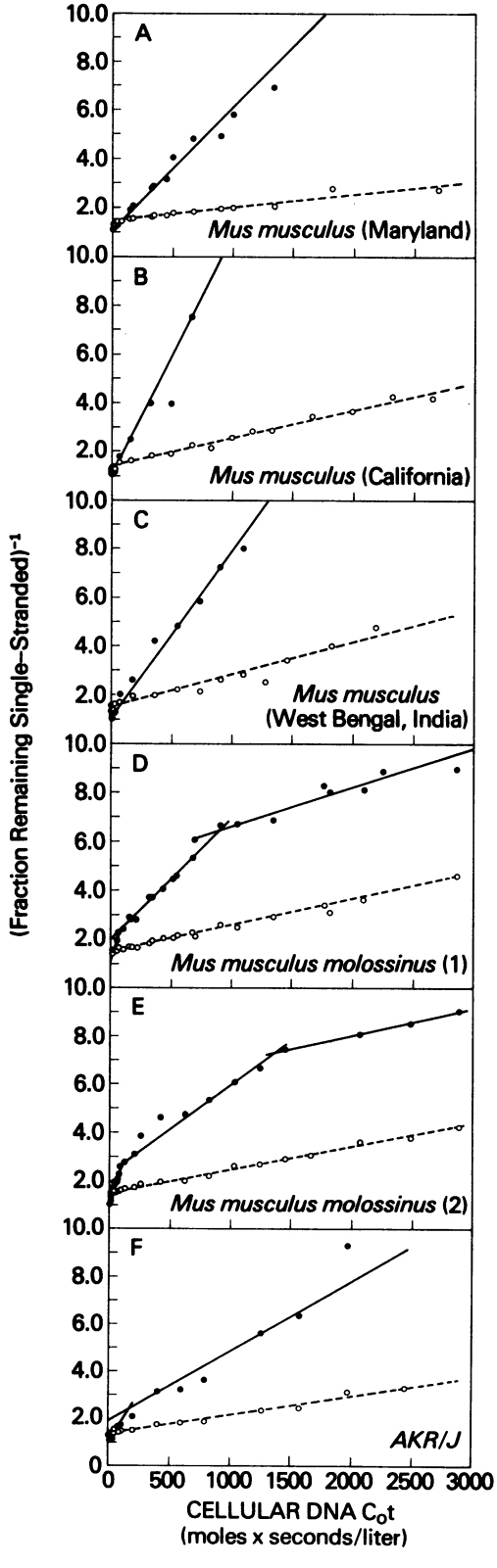


FIG. 1. *Association kinetics of the ecotropic AKR viral [^{3}H]DNA probe with mouse cellular DNAs and corresponding Te_{50} analysis of the hybrids. For annealing reactions, sheared cellular DNAs (10 to 12 mg/ml) were mixed with 1 ng of viral [^{3}H]DNA probe per ml (specific activity, 2×10^7 cpm/μg) in a Reactival (0.3- or 1-ml capacity). The mixture was then denatured in 0.12 M phosphate buffer (PB) by heating at 100°C for 5 min and brought up to desired salt concentrations by the addition of 4.8 M PB. All of the incubation mixtures contained 0.5 mM EDTA. Incubation mixtures with a low salt concentration (0.18 or 0.3 M Na^+) were incubated at 60°C, whereas those with high salt concentration (0.72 to 1.0 M Na^+) were incubated at 65°C. Samples (20 to 25 μl) were taken at different time intervals and diluted to 3.0 ml in a final concentration of 0.14 M PB plus 0.4% sodium dodecyl sulfate (SDS). The extent of hybridization at each time point was assayed by hydroxyapatite chromatography. Unhybridized molecules were removed from the column with 0.14 PB plus 0.4% SDS at 60°C, whereas the hybridized molecules were removed with the same buffer at 100°C. Each fraction eluted from the hydroxyapatite column was measured for absorbance at 260 nm (to measure cell DNA-cell DNA association) and, after addition of 12 ml of Instagel (Packard Instrument Co.) to 8 ml of aqueous solution, for radioactivity (to determine ^{3}H probe-cell DNA association). C_0t values represent the equivalent C_0t at 0.18 M Na^+ (2, 3). In each panel, open symbols represent the association kinetics of viral [^{3}H]DNA with the cellular DNA. Thermal elution profiles were obtained as follows: 100 μl of incubation mixture containing viral [^{3}H]DNA and cellular DNA at the same ratio as mentioned above, in the presence of 0.72 M Na^+, were incubated at 65°C for 72 to 80 h ($C_0t = 4 \times 10^4$ to 5×10^4). Each incubation mixture was then brought to 0.14 M PB plus 0.4% SDS and passed over a hydroxyapatite column (60°C, 0.14 M PB plus 0.4% SDS). Single-stranded DNA was removed from the column with 0.14 M PB plus 0.4% SDS. The temperature of the column was then raised in a series of 5°C increments, and after each increment the column was washed at the new temperature with 8 ml of 0.14 M PB plus 0.4% SDS. Each fraction was measured for absorbance at 260 nm to monitor the elution of cell DNA; after addition of 12 ml of Instagel, the radioactivity in each fraction was determined (to monitor the elution of viral [^{3}H]DNA probe). The graphs present the cumulative proportion of the DNA eluted from the column in relation to the amount bound to the hydroxyapatite at 60°C, as a function of temperature. Open symbols represent the elution profile of cellular DNA self-hybrid molecules, and closed symbols represent the elution profile of viral [^{3}H]DNA-cell DNA hybrid molecules.*



cellular DNA sequences. The third component in the *M. musculus molossinus* DNA curves, not seen with AKR (or any other laboratory mouse DNA), had a slope of 1 and intersected the ordinate at about 5. We interpret these results as showing that there is marked similarity in the relative abundance of subsets of viral sequences in *M. musculus molossinus* and AKR cellular DNAs, but that there is one portion (about 20%) of the probe sequences that is represented by multiple copies in AKR DNA, but only at a copy number of one or two per haploid genome in *M. musculus molossinus*. Although this probe fraction presumably consisted of AKR viral sequences, it must be kept in mind that in cDNA preparations prepared with virus grown in mouse cells, about 20% of the ^3H counts were in nonviral DNA sequences. Use of molecularly cloned viral probes should clarify the nature of this difference.

We also tested *M. musculus molossinus* DNA for the presence of amphotropic 1504-A viral sequences. This is a virus isolated from an embryo of a wild-caught mouse from the La Puente area of California (11, 20); although this class of MuLV is not detectable in laboratory strains of mice, the complete genome sequences are present in cell DNA in all strains, as well as in wild-caught *M. musculus* DNA (5).

The complete genome sequences of amphotropic 1504-A virus were present in *M. musculus molossinus* (Fig. 3A), and the representation of these sequences in *M. musculus molossinus* DNA was the same as in the DNA of previously tested strains (Fig. 3B; 5); there was no evidence of a third component as was seen with the ecotropic virus probe.

DISCUSSION

The data presented in this report provide definitive evidence that N-tropic, ecotropic AKR-type MuLV is present as endogenous DNA sequences in *M. musculus molossinus* and *M. musculus castaneus*, although absent from certain other wild *M. musculus* populations. Using the Southern blotting technique (25) for molecular hybridization of restriction endonuclease-digested cellular DNAs, Steffen et al. (25a) have independently reached similar conclusions. Our results also show that the virus carried in *M. musculus molossinus* DNA is identical to the AKR ecotropic virus in its cleavage

FIG. 2. *Analyses of the association kinetics of [^3H]DNA probe with various mouse cellular DNAs by the reciprocal plotting method (26). The data are from Fig. 1. The maximum observed probe [^3H]DNA-cell DNA and cell DNA-cell DNA hybridizations were normalized to 100%. The symbols are the same as in Fig. 1.*

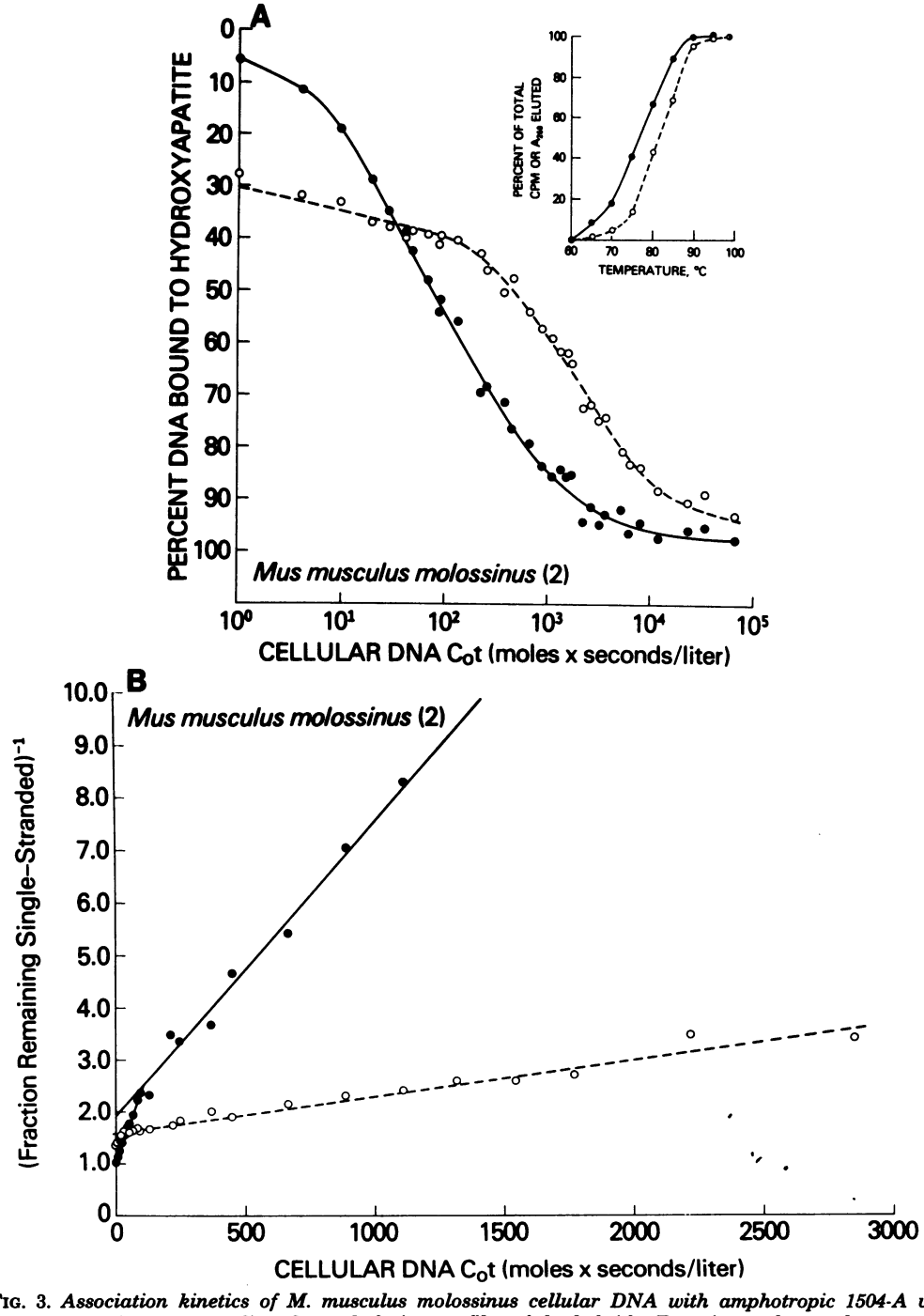


FIG. 3. *Association kinetics of M. musculus molossinus cellular DNA with amphotropic 1504-A viral [^{3}H]DNA and the corresponding thermal elution profiles of the hybrids. Experimental procedures are the same as for Fig. 1 and 2 (A) Britten and Kohne plot (3) of the hybridization data. Open symbols represent self-hybridization of cellular DNA; closed symbols represent hybridization of viral [^{3}H]DNA with cellular DNA. (Inset) Melting profiles of hybrids formed between ^{3}H-labeled viral DNA with the cell DNA and the cell DNA self-hybridized molecules. Symbols are as described above. (B) Reciprocal plot (26) of the data in (A). Symbols are as in (A).*

TABLE 2. *Hybridization of ecotropic and amphotropic viral [^{3}H]DNA to the cellular DNAs*

[^{3}H]DNA from:	Source of cellular DNA	Cell DNA self-hybridization (%)	Viral probe/cell DNA hybridization (%)	No. of sequence populations	Approx no. of copies/haploid genome[a]	ΔTe_{50} (°C)[b]
Ecotropic AKR L1	AKR[c]	95	100	2	>16; 4	2
	NIH Swiss[c]	96	77	1	>10; 0	7
	M. musculus[d] (Maryland)	95	77	1	>12; 0	6
	M. musculus[e] (California)	96	80	1	>6–7; 0	7.5
	M. musculus[d] (West Bengal, India)	96	80	1	>6–7; 0	6
	M. musculus molossinus-1[e]	96	100	3	>16; 4–5; 1–2	2.5
	M. musculus molossinus-2[d]	94	99	3	>18; 4; 1–2	2.6
	M. musculus castaneus[e]	92	98			2.8
Amphotropic 1504-A	*M. musculus molossinus*-2[d]	93	98	2	>20; 8	4.5
	AKR[c]	98	100	2	>10; 6–7	4.0
	NIH Swiss[c]	90	99	2	>10; 5–6	4.0

[a] Determined from reciprocal plot (26; Fig. 2, 3B). The number of copies is the ratio of the slope of each line to the slope of the line described by the unique sequences of cell DNA. Copy numbers were determined by visual fitting.

[b] Difference between the Te_{50} values of self-hybridized cell DNA molecules and that of probe-cell DNA hybrids.

[c] From embryos.

[d] From tissues.

[e] From tissue-cultured cells.

patterns with seven restriction enzymes and that RNA from this virus hybridizes as extensively to the AKR MuLV cDNA probe as does RNA from AKR virus.

It has long been known that Japanese mice are among the ancestors of laboratory mice (13, 24), and the present findings are consistent with this fact. Even if laboratory mice include *M. musculus molossinus* among their ancestry, the two types of mice must now be separated by thousands of generations; the degree of conservation of endogenous viral sequences, which are not known to supply positive selection value, seems striking. Further comparisons of the organization and chromosomal distribution of ecotropic and other viral sequences in *M. musculus molossinus* DNA may be useful for understanding the factors affecting the stability and plasticity of endogenous viral sequences.

The finding of a different proportion of viral sequence populations in the *M. musculus molossinus* DNA was unexpected. Of the more than 20 inbred strains and wild-caught *M. musculus* whose DNAs we have studied, only *M. musculus molossinus* showed this pattern. It will be of interest to study this problem with subgenomic fragments.

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We thank Michael Potter for providing us with live *M. musculus molossinus* and *M. musculus castaneus* mice, T. H. Yosida for frozen *M. musculus molossinus* tissue, A. S. Levine and Janet W. Hartley for support and encouragement.

ADDENDUM IN PROOF

Since submission of this manuscript, we have generated a restriction endonuclease map of *M. musculus molossinus* ecotropic virus by using 12 enzymes. The pattern is identical to that of the *AKv-1* isolate (E. Rands, D. R. Lowy, M. R. Lander, and S. K. Chattopadhyay, Virology, in press).

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